University of South Bohemia in České Budějovice

Faculty of Science

Eating the cold air: Isolation and identification of aerobic psychrophilic diazotrophs from permafrost soil.

Bachelor thesis

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Annotation:

Potential aerobic psychrophilic diazotrophs were isolated from subarctic degraded permafrost soils (wet and dry conditions) and a control (intact condition) using Norris Glucose Nitrogen Free Medium under the two different temperatures. DNA of singular bacterial colonies was extracted followed by 16S rRNA gene amplification and sequencing, to identify the soil isolates at genus level. The extracted DNA was further used to amplify the *nifH* gene.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 08.05.2024

Acknowledgement:

Firstly, I would like to thank Jiří Bárta, for being my supervisor and allowing me to be part of this research. Next, I would like to thank Muhammad Waqas for assisting me in the lab as well as with the writing process. Lastly, I would also like to thank Eva Koutecká and Monika Strejčková for helping me obtain materials and navigate machines in the lab.

Abstract:

Psychrophilic diazotrophs are nitrogen fixing bacteria capable of living in low temperature soils, such as in degraded permafrost, and they are characterised by containing the *nifH* gene. With the increase of permafrost thawing, there is an increase of nitrogen released from the previously sequestered nitrogen stores in the soil. Studying microorganisms that fix this nitrogen can contribute to a wider understanding of the biochemical process in the thawing soil. In this study, bacteria were isolated from degraded subarctic permafrost from a wet and dry landscape, as well as a control intact soil. The bacterial isolates were incubated at two different temperatures and their DNA was sequenced to obtain information about their taxonomy. The *nifH* gene was also amplified through PCR. Overall, the soil isolates were split into eleven different genera and three of the bacterial species were positive for the *nifH* gene.

List of abbreviations:

% - percentage

ANRA - assimilatory nitrogen reduction to ammonia

Bp - base pairs

BSA - bovine serum albumin

C - Carbon

cm - centimeter

ddH_2O - distilled deionized water

DNRA - dissimilatory nitrogen reduction to ammonia

DNA - deoxyribonucleic acid

EPS - exopolysaccharides

g - gram

gr - gravity

H - hectare

HCl - Hydrochloric acid

kg - kilogram

L - liter

M - molar concentration

min - minute

µl - microliter

N - nitrogen

N_2 - nitrogen gas

N_2O - nitrous oxide

NC - negative control

NH_3 - ammonia

NH_4+ - ammonium

NGNFM - Norris Glucose Nitrogen Free Medium

O_2 - oxygen

p - potential hydrogen

PCR - polymerase chain reaction

PC - positive control

pH - potential hydrogen

rpm - rotations per minute

rRNA - ribosomal ribonucleic acid

sec - second

V - volts

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Introduction

Permafrost

Permafrost is a layer of soil that remains frozen during the year, having temperatures which do not exceed 0 °C for a duration of at least two years, covered by an active layer which thaws seasonally (Riseborough et al., 2008). Due to the absence of thawing, it acts as a storage, with nutrients sequestered in it trapped almost indefinitely. Frozen water prevents runoff and in these low temperatures organisms are also preserved, but they are inactive. These nutrients and microorganisms are gradually stored over thousands of years, largely unaffected, however with the change in temperature due to climate change, permafrost is starting to thaw, releasing nutrients which drive the biogeochemical cycles, and reviving latent microbes. The thawing occurs from the upper layer downwards, so the more recently sequestered material is released first, but as the thawing increases in depth, so does the age of the stored compounds and organisms within. This change in nutrient cycling is predicted to largely alter the arctic ecosystems (Dobiński, 2020; Miner, 2021).

The uppermost layer of permafrost is the so called “active layer”, which is the location for most ecological and biochemical processes in permafrost soils and is repeatedly thawing and freezing every season. The active layer may vary in thickness, from a few cm to a meter and more in warmer areas or areas where the degradation of permafrost is the highest (Van Everdingen, 1998; Dobiński, 2020). The two main permafrost thaw conditions, which can lead to the extension of the active layer, are the wet and the dry landscape These are two extreme contrasting environmental conditions, where the soil is either water-saturated, which can be seen in soils that have an abundance of ice wedges (wet landscape), or where the soil is dry, due to improved drainage and evapotranspiration (Natali et al., 2015). The two types of soil can be seen in Figure 1.

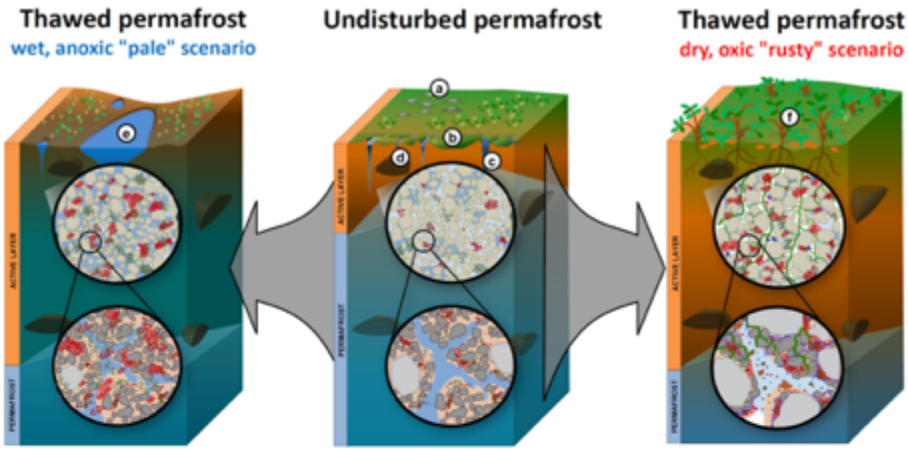

Figure 1. Two contrasting conditions of soil development with permafrost thawing. Thawed permafrost with the wet and anoxic condition has less vegetation and roots are restricted to topsoil while dry and oxic condition has more vegetation and a deep rooting system.

Nitrogen in thawed permafrost

Global warming is the leading cause of largescale permafrost thawing, altering nutrient availability and cycling patterns in some affected areas. There is a large abundance of nitrogen (N) in permafrost soils, mainly stored in organic forms in microbial biomass or in inorganic forms within frozen soil (Jonasson et al., 1996). Upon permafrost thawing, some N may become immediately available for the plants, however the remainder, which is stored inorganically, can enter the ecosystem through gradual mineralization accomplished by microorganisms (Keuper, 2012).

The active layer of permafrost which freezes and thaws periodically is the location of the N competition zone in permafrost environments. Plants rely heavily on decomposition for a source of N as there is little deposition of N and N_2-fixation rates are low in such an environment, however the cold temperature significantly hinders the rate of decomposition (Ping et al., 2015; Weintraub & Schimmel, 2003). N is a limiting nutrient for plant growth in the arctic regions as not only is there less profound atmospheric deposition but also restricted N_2-fixation which ranges from 0.65 h^{-1} kg^{-1} to 10 h^{-1} kg^{-1}. For proper utilization of the released N, plant roots must reach sufficient depths or the N must shift to the shallower layers (Hansen and Elberling, 2023). There is an abundance of bioavailable N in permafrost, however it is sequestered and not released unless the permafrost melts. Although upon melting, there is an increase in plant uptake of N, a lot of this

available N is also associated with runoff as the melting water transports it from the soil into nearby water reservoirs. After thawing, the already limited N may be leached out of the ecosystem, either by dissolving in water and being transported to aquatic ecosystems or lost in the form of nitrogen gas (N_2) and nitrous oxide (N_2O) as a result of denitrification (Hansen and Elberling, 2023). Exact models of N transport after thawing are yet to be mapped (Salmon, 2018).

Modelling predictions of carbon (C) cycling also depends on N availability, as this element determines the rate of plant productivity and decomposition (Hansen and Elberling, 2023). Furthermore, the movement of nutrients is expected to change alongside a change in rainfall dynamics, as there is a prediction of more liquid compared to snow precipitation in permafrost regions in the future (Meredith et al., 2019).

Nitrogen cycle

N is a necessary component of biological compounds including proteins and nucleic acids, thus its proper availability and cycling is important for sustaining life in ecosystems (Bloom, 2015). The N cycle, as seen in Figure 2. comprises three main parts: N_2 fixation, nitrification and denitrification. These terms are also used as identifiers for the microorganisms carrying out the respective function. These three components can also be further divided into five main N transformation flows: ammonification (comprised of N_2-fixation and assimilatory (ANRA) and dissimilatory (DNRA) reduction of nitrite), nitrification, denitrification, anammox (coupled nitrification-denitrification) and lastly nitrite-nitrate interconversion. The compounds ammonia (NH_3) and ammonium (NH_4^+) become incorporated in biomass through assimilation and leave through mineralization (Stein & Klotz 2016).

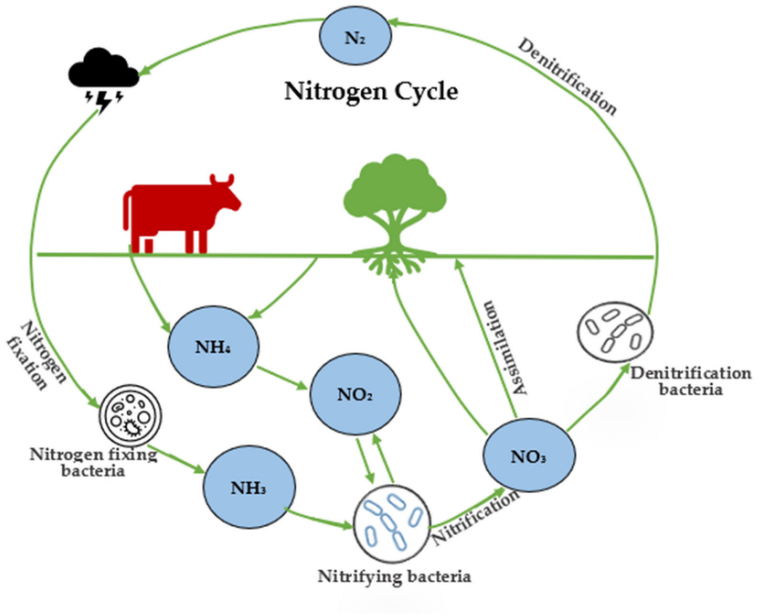

Figure 2. Nitrogen cycle (Shakil et al., 2022)

The first transformation of N_2 which contributes to it being assimilated is the aforementioned ammonification which has two main processes. N_2-fixation, which is done by bacteria and archaea that produce the nitrogenase enzyme. This enzyme is a ferrous complex and it is sensitive to oxygen (O_2), thus the organisms must find a way to maintain this as an anaerobic process, mainly by adapting as anaerobic or facultatively anaerobic. ANRA and DNRA is done by bacteria and fungi and it is essentially the nitrate reduction to nitrite, DNRA is activated by a negative redox potential, while ANRA is facilitated by N reductases. To make this fixed N even more available, nitrification is achieved by three main groups of organisms. First ammonia oxidizers oxidize ammonia to nitrite, then nitrite oxidizers oxidize this to nitrate or complete ammonia oxidizers oxidize ammonia straight to nitrate. Lastly, denitrification occurs which is a type of anaerobic respiration which releases N_2 and consumes NO_2^-, NO, N_2O. This way the N may remain balanced and maintain soil health (Stein & Klotz 2016).

Psychrophilic Diazotrophs

Many microorganisms have evolved to be able to survive in various extreme environments, an example of this are psychrophiles, which live in permanently cold temperatures. The majority of the biosphere has a constant temperature below 5 °C, this includes the deep sea, glaciers and snow and permafrost covered land. To survive in such conditions, organisms developed structural and functional adaptations to counteract issues such as reduced enzymatic rates, limited bioavailability of nutrients, extremes in pH and salinity, freeze-thaw cycles and low soil moisture (Morita, 1975; Margesin & Miteva, 2011).

Permafrost coverage accounts for about 15% of the Earth's land surface in the Northern Hemisphere and has a varied range in temperature, from around 0 °C to -10 °C and lower (Obu, 2021; Romanovsky et al., 2002). Depending on the location permafrost contains 20-70% of ice and only about 1%-7% unfrozen water. In terms of microbial diversity, Arctic permafrost soils contain mainly gram-negative bacteria, specifically *Pseudomonas* which can generally makeup 60% of the bacterial diversity as well as *Acidobacteria* in low pH soils (Männistö & Häggblom, 2006).

One of the key advantages of psychrophilic organisms in general is to be able to adapt to changes in temperature by modifying their cell membrane, and therefore influence the rigidity (e.g. saturation of fatty acids, etc.). Other processes which must be improved are maintaining membrane fluidity, production and acquiring of cryoprotection compounds, antioxidant activities and support of protein synthesis and protein folding. Cryoprotection compounds minimize the negative effects of freezing and the formation of ice crystals. The microorganisms also have cold-active enzymes which are more active at low temperatures compared to their regular homologues, but they may become inactivated at higher temperatures (Margesin & Miteva, 2011).

Diazotrophic microorganisms live in both aquatic and terrestrial environments, including extreme environments such as cold arctic/antarctic environments, in the soil they either exist freely or in association with plant roots (Sun, Shahrajabian & Cheng, 2021).

Their presence in soil is affected by various factors, such as pH, moisture, O_2 availability as well as nutrient presence, including N, which can have both positive and negative effects on diazotroph communities (Hsu & Buckley, 2009).

Diazotrophs are N-fixing organisms, meaning they can reduce atmospheric N gas into ammonium, which can be accessed by plants and other microorganisms. N_2 fixation is a key process as it represents the entry of N in an available form of NH_4^+ into the ecosystem (Postgate, 1970). They do this using the enzyme nitrogenase. For their detection and quantification in the environment, the *nifH* gene is often used as a marker (Nash et al., 2018). This gene codes for an iron only subunit of nitrogenase, however, the enzyme itself often contains molybdenum and there are also instances of vanadium based and iron-only nitrogenases (Ribbe, Hu, Hodgson & Hedman, 2014). Its practicality stems from it not only being a highly conserved region among prokaryotes but also being the most currently studied nitrogenase gene. Nitrogenase is sensitive to O_2, so diazotrophs vary from anaerobic to aerobic, with divergent ways of coping with O_2 presence. Most of the N in the high Arctic comes from biological N_2-fixation, while this process contributes to only around a third of the N source in tropical forests (Izquierdo & Nüsslein, 2006).

N is a limiting factor for both plant production and microbial decomposition in cold environments as they share the same N pool. Both free-living and plant-associated diazotrophs are vital to this arctic environment as they supply N to the plants and sustain the productivity of the ecosystem. With the increase in temperatures, deeper layers of soil are experiencing a boost in *nifH* gene occurrence, a gene associated with N_2-fixation. This may be due to not only this environment having an increased temperature, but also a lack of O_2, which is favoured by the anaerobic and facultatively anaerobic bacteria (Feng, 2019).

Alongside bacterial cells and soil organic matter particles, soil is also consistent of other organic matter, such as bacterial capsules. (Campbell, 2009). Many diazotrophs are exopolysaccharide (EPS) forming, such as *Nostoc*, *Burkholderia* etc. (Otero & Vincenzini, 2003; Mattos, et al., 2001). In a thawing arctic environment, EPS is important for holding the soil aggregates together to reduce erosion processes as well as providing a more stable environment for soil bacteria (Schmidt, et al., 2008). These in turn influence soil moisture, as well as create the initial C and N pools after

permafrost soil thaw (Jung et al., 2018). EPS also helps diazotrophs to keep O_2 levels low to protect nitrogenase and increase N_2 fixation efficiency (Sabra, Zeng, Lunsdorf & Deckwer, 2000).

Overall, diazotrophs may serve many roles in degraded permafrost, being vital N_2 fixers that supply available N for the ecosystem, as well as contributing to the overall properties of the soil. Investigating the different diazotroph communities present in various thaw landscapes may uncover more information about the effects of thawing on soil biochemical processes and communities.

Aims

The aims of the thesis were: (i) to isolate psychrophilic diazotrophs from degraded permafrost soil, (ii) taxonomic characterization of the isolated psychrophilic diazotrophs and (iii) to determine the relative proportion of the isolated diazotrophs in total microbiome.

Methods

Soil Sample

Topsoil samples used for diazotroph isolation were taken in two degraded permafrost landscapes: dry and wet and also from non-degraded intact landscapes. Samples were obtained from permafrost sites in Fairbanks, Alaska, USA in 2022. Approximately 2 grams of each type of soil were used for bacterial isolation.

Media preparation

In order to isolate diazotrophs, a selective Norris Glucose Nitrogen Free Medium (NGNFM) was used, composed of nutrients that are all free of N to promote the selection of diazotrophs (Stockdale, Ribbons and Dawes, 1968). The ingredients listed in Table 1. were all added into a bottle with the appropriate amount of distilled water and mixed using a stirrer. While being mixed, the pH was adjusted to be between 7 - 7.2 using 1M HCl and the medium was sterilised in an autoclave for 20 minutes at 121°C. In this study both liquid and solid media were used to isolate diazotrophs. The table below represents solid medium, for the liquid the same recipe was followed with no agar addition.

Table 1. Composition of selective NGNFM N free medium

Chemical	**Amount (g/L)**
Glucose	10
Dipotassium hydrogen phosphate	1
Magnesium sulphate	0.2
Calcium carbonate	1
Sodium chloride	0.2
Sodium molybdate	0.005
Ferrous sulphate	0.1
Agar	20

Serial dilutions of soil samples

Approximately 2 g of soil sample (done for all soil samples from dry, wet and control intact) was added to a sterile 50 ml falcon tube and mixed with 18 ml sterile saline solution. The falcon tube was then vortexed for 5 minutes and left to rest for 2 minutes. Following this, a dilution series was prepared, with a 1:10 dilution ratio, which was done by combining 100 µl of dissolved soil with 900 µl of saline solution in an Eppendorf tube and repeated until a 10^{-5} dilution was reached.

Inoculation

At first, 100 µl of the 10^{-2} dilutions were added to 10 ml liquid NGNFM under sterile conditions. This was done in triplicates, where three falcon tubes from each sample (wet, dry and intact control) were incubated on a shaker at 180 rpm in the two conditions 10 °C and 22 °C, for four days. Barely any growth was observed and on microscopy it proved difficult to find bacteria, with growth being present but only very minimal. For this reason, the method was altered. To move the bacteria onto plates, 100 µl of the previously liquid culture was poured onto NGNFM agar, each falcon tube receiving a corresponding agar plate. Additionally, 100 µl from the 10^{-3} serial dilution was also plated, with the same method as mentioned above with triplicates of each sample being made for every corresponding condition.

Microscopy

Microscopy for investigating bacterial cells after inoculation to liquid culture was done using the Gram staining method (Bartholomew & Mittwer, 1952). The Gram staining method involved taking a few drops of solute and placing it onto a microscopy slide, using an inoculation loop the drop was spread on the slide to attempt to separate the culture of bacteria to see singular bacterial cells more clearly. The bacterial culture was then heat fixed onto the slides and left to air dry. After air drying crystal violet was added to the slide and let sit for about a minute. This was then washed using iodine solution for about a minute and then decoloured with ethanol. The last step involved adding safranin and leaving for around 45 seconds. Then the slide was washed with distilled water to remove the excess colour and after drying it was examined under a microscope (Olympus BX61, Japan) with 1000x magnification using immersive oil and a UPlanApo 100x/1.35 Oil iris lens and pictures were taken using the Canon DS126571 camera with the EOS utility ver.3.

Isolation of single colonies

After the incubation of diluted agar plates, a well-grown single colony was picked and streaked on fresh NGNFM agar plates under sterile conditions. The agar plates were then incubated in either 10 °C or 22 °C depending on under which temperature the colony was initially cultivated. Following the initial incubation, the streaking process was repeated a minimum of three times to isolate pure colonies.

Collection of colonies from diluted agar plates

Approximately 1 ml of sterile saline solution was added to agar plates and using a sterile inoculum loop the colonies were mixed and detached from the medium. Then the colony mixture was transferred into a sterile Eppendorf tube and stored at -20 °C.

DNA extraction

DNA extraction was done by following the method included in the DNeasy UltraClean Microbial Kit (250). The collected pure strain colonies from agar plates were centrifuged at 13000 gr for 1 min at room temperature, which were the default settings for all the following centrifugation steps. The supernatant was decanted and centrifugation was repeated, with the remaining supernatant being carefully removed with a pipette so only the pellets remained, which were then resuspended in 300 µl of PowerBead solution and relocated into PowerBead Tubes. Then 50 µl of SL solution was added to the tubes. The tubes were horizontally fixed into a beadbeater and vortexed at maximum speed for 30 sec. Afterwards, the tubes were centrifuged and the supernatant was transferred into a clean 2 ml collection tube. After the addition of 100 µl of IRS solution, the tubes were mixed by shaking and incubated on ice for five minutes. The tubes were centrifuged again and the supernatants were moved to new collection tubes, where 900 µl of Solution SB was added before an additional 5 sec vortexing. Around 700 µl were loaded into an MB Spin Column and centrifuged, after the supernatant was removed, this was repeated with the remaining supernatant from the previous step. Then 300 µl of CB Solution was added and the tubes were centrifuged once again, and then centrifuged once more after the flow-through was discarded. Next, the Spin

Column was moved to a new 2 ml collection tube and 30 µl of EB Solution was pipetted directly onto the white membrane. Lastly, the tubes were centrifuged, the spin column was discarded and the extracted DNA was stored under -20 °C.

DNA quantification

For DNA quantification two solutions, the 1x TE buffer and Quantifluor Dye working solution were prepared in a low light environment, according to Table 2. below, which corresponds to one sample, the amount was adjusted according to how many DNA samples were being quantified.

Table 2. DNA quantification solution preparations

1x TE buffer		QuantiFluor Dye working solution	
20X TE (µl)	H2O (µl)	QuantiFluor Dye (µl)	1X TE buffer (µl)
10.00	190.00	0.5	99.5

After the solutions were prepared, 2ul of DNA was mixed with 98 µl of 1x TE buffer and 100 µl of QuantiFluor Dye working solution in a 1 ml PCR tube, the same recipe was used to make a standard solution, except 2ul of STD were used instead of DNA. A blank was also prepared by mixing 100ul of 1x TE buffer with 100ul of QuantiFluor Dye working solution. The tubes were incubated in the dark for 10 min and then analyzed using the Quantus fluorometer.

Molecular characterization of isolated diazotrophs

To analyze 16S rRNA gene, PCR was performed using a mix consisting of the chemicals in Table 3. The primers used were 9bfm (forward primer 5'-GAGTTTGATYHTGGCTCAG-3') and 1512uR (reverse primer 5'-ACGHTACCTTGTTACGACTT-3'). For a positive control (PC) DNA of *Escherichia coli* (strain ATCC 9637) and for a negative control (NC) H_2O were used. The PCR amplification was carried out using the following cycle: initial denaturation for 5 min at 98 °C, followed by 30 cycles each for 30 sec at 98 °C (denaturation), 30 sec at 62 °C (annealing) and 90 sec at 72 °C (elongation). The final elongation was carried out at 72 °C for 10 min.

Table 3. 16S rRNA PCR master mix for bacterial isolate identification, ddH_2O (distilled deionized water), BSA - (bovine serum albumin)

All bacteria, PCR	
No. of samples	**1**
volume	**25**
	1x (µl)
ddH_2O water	10.7
FastPCR buffer	12.5
9bfm (F primer)	0.25
1512uR (R primer)	0.25
BSA	0.3
Total volume	
pipet	24 µl
DNA template	**1 µl**
Negative control	H_20
Positive control	*E.coli*

PCR conditions for *nifH* gene amplification

To amplify the *nifH* gene in the DNA of soil isolates, the cycle and chemicals as seen in Table 4. were used. The forward primer is IGK3 (GCI WTH TAY GGI AAR GGI GGI ATH GGI AA) and the reverse primer is DKK (ATI GCR AAI CCI CCR CAI ACI ACR TC). (Ando et al., 2005). A mastermix was prepared according to this table and then the concentration of DNA was adjusted so that 2 ng were always being added to the mix, if the amount required a higher or lower amount of DNA template to be added, the volume was adjusted using water, to ensure that each tube had 25 µl. The PCR cycle used was as follows: initial denaturation for 10 min at 95 °C, followed by 30 cycles each for 45 sec at 95 °C (denaturation), 30 sec at 58 °C (annealing) and 60 sec at 72 °C (elongation). The final elongation was carried out at 72 °C for 10 min. The negative control used was H2O and the positive *Azotobacter vinelandii* (DSM 2289).

Table 4. PCR mastermix for *nifH* gene amplification

No. of samples	**1**	
Total µl	**25**	
	One sample µl.	Conc.
water	16.825	
buffer	2.5	1x
dNTP	0.5	
IGK3	0.25	100 µM
DVV	0.25	100 µM
BSA	0.3	
DMSO	0.625	
Taq	0.25	
$MgCl_2$	2.5	
Volume of MM	25	
Pipette	24	
DNA template	1	

Gel electrophoresis

To test whether the PCR amplification was successful, the PCR products underwent gel electrophoresis on a 1% agarose gel. This was done by dissolving 1% of agarose in 1x TAE buffer, such as 1g in 100 ml in an erlenmeyer flask. To fully dissolve, the flask was placed in a microwave for seven minutes. After cooling until no longer scalding to touch, 3 µl of EliDNA were added, mixed and the solution was poured into a gel tray and left to solidify covered by aluminium foil. When completely solid, the gel was loaded with 4 µl of PCR product that was mixed with 1ul of agarose dye. The first well was filled with 5 µl 1kb DNA ladder. When loaded, the gel was run at 120 V, 500 rpm for 50 min. The gel was visualised by a transilluminator (Azure 200, Azure Biosystem, Inc, US).

PCR product purification

The final PCR product was purified using the QIAquick® PCR Purification Kit. First 225 µl of PB Buffer were added to the 25 µl of PCR product and mixed using a pipette. A QIAquick column

was placed into a 2 ml collection tube, and the initial mixture was placed into the QIAquick column. This was centrifuged at 13000 gr for 1 min at room temperature (this was again the set up for further centrifugation steps) and the flow through was discarded. Then 750 µl of PE Buffer was added and the collection tube with the column was centrifuged. The centrifugation was repeated one more time. Then the QIAquick column was placed in a clean 1.5 ml collection tube and 50 µl of EB Buffer was added directly to the membrane. After one last centrifugation, the column was discarded and the purified PCR product was stored at 4 °C. The concentration of the PCR product was checked using the same method as for DNA.

Sequencing

Following PCR purification, the samples were sent for sequencing to the SEQme sequencing company (Prague, Czech Republic). The sequencing data received from the sequencing company was then trimmed, cleaned, and identified using BLASTn in Geneious prime (Altschul et al.,1990).

Microscopy for bacterial examination

To observe the pure soil isolates via microscopy, a small amount of a colony was mixed with distilled water on a microscopy slide using a sterilised inoculum loop. This slide was heat fixed by briefly placing it three times under a flame. To dye it, Hiss's staining method was adapted: the slide was covered with 0.1% crystal violet and washed with 20% copper sulphate and then with distilled water. (Hiss, 1905) After the slide dried, a drop of immersion oil was placed onto it and the slides were visualised under the microscope.

Determination of the proportion of diazotroph isolates in total bacterial community

To determine the relative abundance of the diazotroph isolates in the total bacterial community of permafrost soil (dry, wet and intact control), 16SrRNA gene sequences from the isolates were blasted against a local database constructed from 16SrRNA sequences of operational taxonomic units (OTUs) from the total microbiome from 2021 of permafrost soil. The threshold for the similarity between sequences was set to >95%. For those OTUs that were more than 95% similar

to the pure isolates, relative abundances at each site were calculated according to the OTU table. The proportion of isolated diazotrophs among the total bacterial community was statistically analysed by R Core Team 2020 (R v 4.0.2) using packages Phyloseq, Vegan and Tidyverse. The results are displayed in Figure 8. which was generated via ggplot2 (Wickham, 2016).

Results

CFU calculation

The colony forming unit (CFU) was calculated to determine the number of colonies per gram of soil sample. This was done only with the original 10^{-2} plates of 22 °C since the colonies on the 10 °C plates did not have sufficient amounts of colony growth. Total colonies were counted on poured plates initially after 20 days of growth and then after an additional seven days. This amount was then averaged, and taking into account inoculation volume, dilution, as well as sample mass, the CFU per gram was calculated.

Table 5. CFU count table

Condition/ 22 °C	Mean/ # of colonies	CFU/g of soil
Wet	77	37800
Intact	12	6000
Dry	2	995

The lack of, or very low growth of diazotrophs at 10°C could be connected to low activity of nitrogenase at this temperature (Waughman, 1977). The disparity between the conditions also suggests that wet active site soil is the most hospitable to a wide range of colony forming cells.

Isolation of colonies

Ultimately, 23 of the colonies from the plates which were incubated at 22 °C and 10 colonies from the 10 °C plates were picked and streaked several times to ensure a pure colony (Figure 3.). For

the 22 °C plates, the distribution was 13 wet, 4 dry and 6 intact (control). The 10 °C plate distribution consisted of 5 wet, 3 intact and 2 dry.

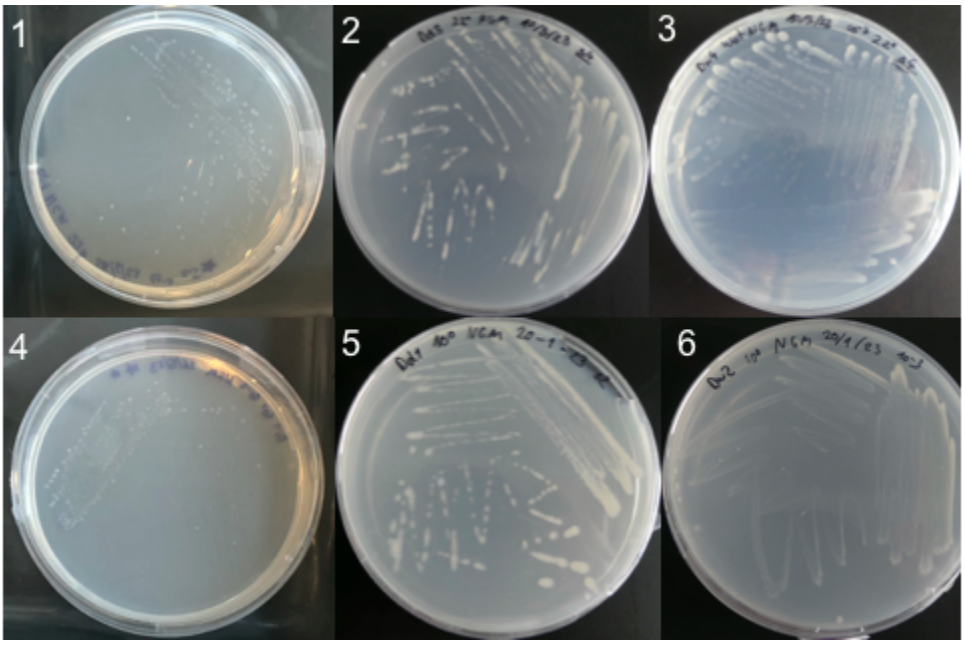

Figure 3. Inoculated bacterial plates

Plates show the following bacterial diazotroph isolates: 1. *Paenibacillus sp.* 2. *Paraburkholderia sp.* 3. *Phyllobacterium sp.* 4. *Paenibacillus sp.* 5. Sequencing not successful 6. *Arthrobacter sp.*

Taxonomic characterisation of isolated diazotrophs

According to the PCR using universal bacterial primers 9bfm (forward primer) and 1512uR (reverse primer) for the 16S rRNA gene amplification, the resulting PCR products have the expected size of the 16S rRNA gene, which is approximately a 1.5 kb DNA fragment. An example of the gel layout can be seen in Figure 4. In this particular gel, all the samples successfully not only contained a sufficient concentration of the 16S rRNA, but the bands of the samples also corresponded to the band of the positive control, with all of them being at around 1500 bp. All samples which contained a visible band on this PCR were sequenced.

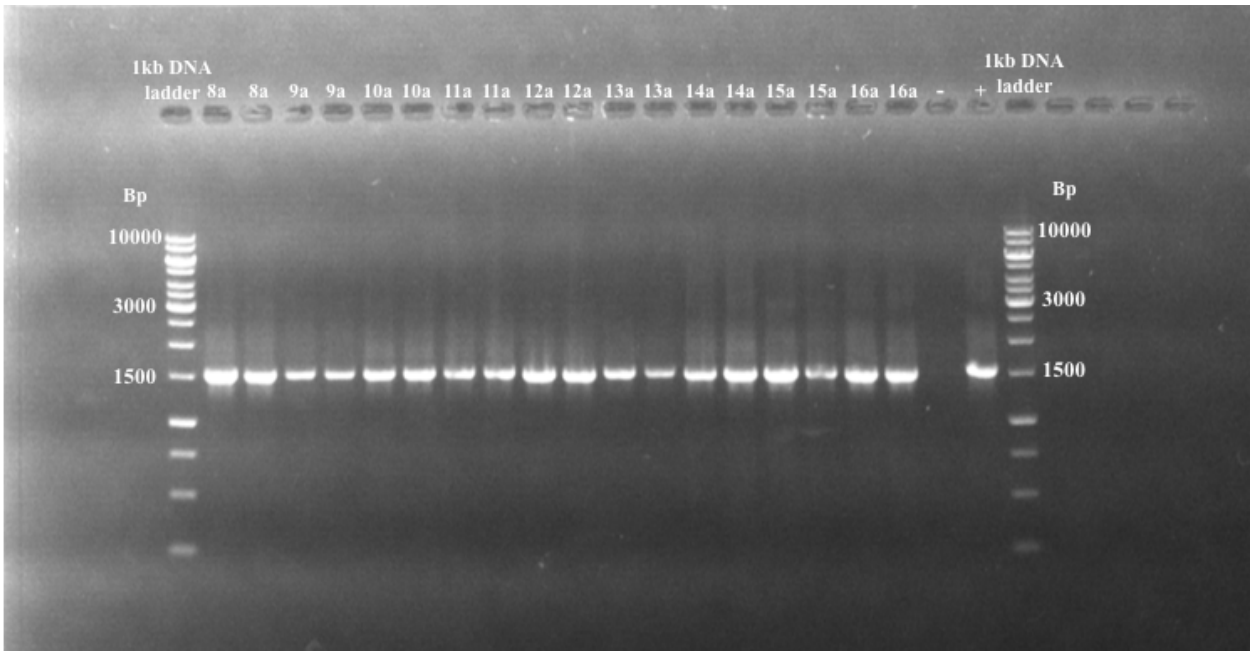

Figure 4. Agarose gel analysis of amplified 16S rRNA gene.

Sequence analysis of 16S rRNA amplification

The results from the 25 sequenced samples are shown in Table 6. (the second letter in the Sample ID indicates site condition (d = dry, i = intact, w = wet)) and the proportion of these results for each landscape can be seen in Figure 5. The isolates which have less than 95% similarity are in quotation marks, as the genus could not be accurately identified and therefore the similarity was restricted to Order level. According to nucleotide sequence data from NCBI BLASTn, the genus *Paenibacillus* was the most present, followed by *Pseudomonas* and *Paraburkholderia*. In dry soil conditions, only *Paraburkholderia* were present. In wet soil, *Paenibacillus* and *Pseudomonas* were predominant, followed by *Peribacillus, "Flavobacterium"* (Order: *Flavobacteriales*) and *Rhizobium* (Order: *Hyphomicrobiales*), while *Arthrobacter* and *Phylobacter* had one strain each. In intact soil, *Paenabacillus* was dominant with two strains, while the other genera present; *Ameyamaea, Pseudoarhtrobacter, Mycolicibacterium, "Kocuria"* (Order: *Micrococcales*) and *Peribacillus* had one strain each.

Table 6. Overview of bacterial isolates and BLAST results against NCBI 16SrRNA gene database

Sample ID	**Temp. °C**	**Landscape**	**Pairwise Identity**	**Identical Site**	**Order**	**Genus**
Dd1	22	Dry	99.20%	99.20%	Burkholderiales	*Paraburkholderia*
Di1*	22	Intact	98.80%	98.80%	Bacillales	*Paenibacillus*
Dw3	22	Wet	99.60%	99.60%	Hyphomicrobiales	*Rhizobium*
Dw2a	22	Wet	89.40%	81.30%	Flavobacteriales	*"Flavobacterium"*
Dw4*	22	Wet	99.90%	99.90%	Hyphomicrobiales	*Phyllobacterium*
Di4	22	Intact	97.60%	97.60%	Rhodospirillales	*Ameyamaea*
Dw1	22	Wet	97.40%	97.40%	Pseudomonadales	*Pseudomonas*
Dw2*	22	Wet	99.90%	99.90%	Bacillales	*Peribacillus*
Dw6*	22	Wet	98.80%	98.80%	Bacillales	*Paenibacillus*
Dw3a*	22	Wet	98.70%	98.70%	Bacillales	*Paenibacillus*
Di1a	22	Intact	99.20%	99.10%	Micrococcales	*Pseudarthrobacter*
Dw5a*	22	Wet	99.95%	99.90%	Bacillales	*Peribacillus*
Dd2	22	Dry	97.90%	96.90%	Burkholderiales	*Paraburkholderia*
Dd3	22	Dry	99.30%	99.30%	Burkholderiales	*Paraburkholderia*
Dw2b	22	Wet	87.30%	87.30%	Flavobacteriales	*"Flavobacterium"*
Dd4	22	Dry	97.80%	97.70%	Burkholderiales	*Paraburkholderia*
Dw1*	22	Wet	98.60%	98.60%	Bacillales	*Paenibacillus*
Di1b	22	Intact	98.40%	98.30%	Mycobacteriales	*Mycolicibacterium*
Di2*	22	Intact	99.90%	99.90%	Bacillales	*Peribacillus*
Dw4	22	Wet	93.60%	93.60%	Hyphomicrobiales	*"Rhizobium"*
Dw2*	10	Wet	100%	100%	Micrococcales	*Arthrobacter*
Di1*	10	Intact	99.50%	99.50%	Bacillales	*Paenibacillus*
Di2a	10	Intact	81.90 %	81.90 %	Micrococcales	*"Kocuria"*
Dw3	10	Wet	95.20%	95.20%	Pseudomonadales	*Pseudomonas*
Dw1a	10	Wet	98.80%	97.20%	Pseudomonadales	*Pseudomonas*

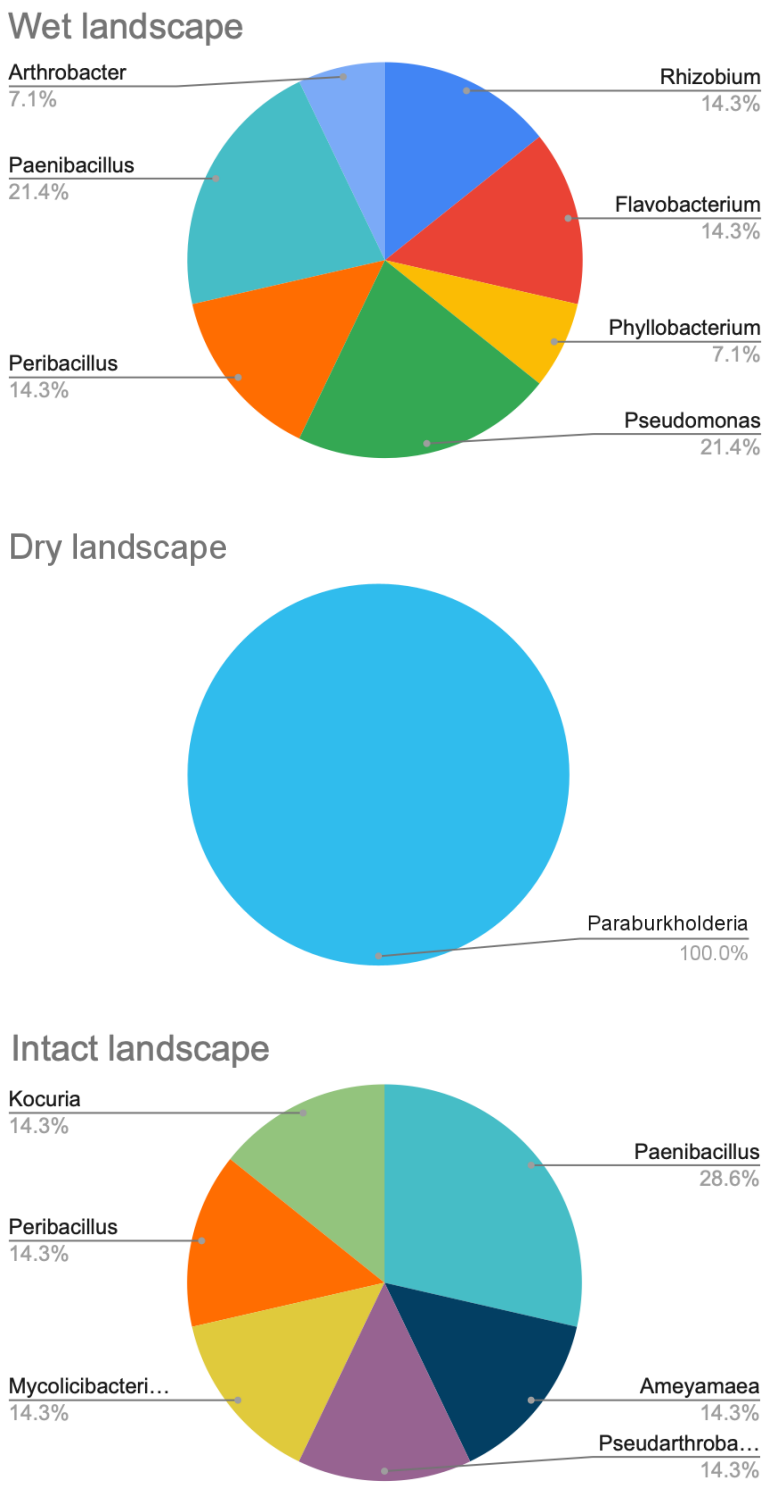

Figure 5. Distribution of bacterial isolates of different genera across different landscapes

nifH gene PCR amplification

To analyse the *nifH* gene in the genomic DNA of the isolates, PCR was performed to amplify the *nifH* gene using the IGK3 and DVV primers. The expected size of the band was compared to the 1kb DNA ladder. According to the *nifH* gene PCR amplification analysis, a fragment about the size of the *nifH* (300 bp) was found only for these isolates: 4b potentially "*Kocuria sp."* (Order: *Micrococcales*), 5b *Pseudomonas asturiensis,* 10b *Pseudomonas syringae*. These can be seen in Figure 6. and 7.

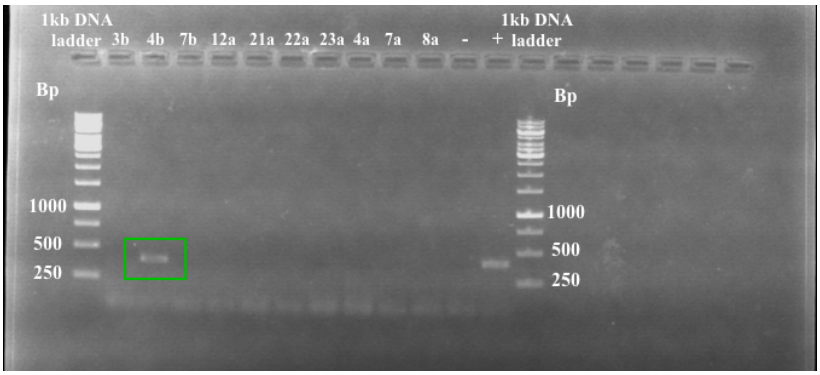

Figure 6. Agarose gel analysis of amplified *nifH* gene (one positive band approx. 300pb for 4b "*Kocuria sp."* (Order: *Micrococcales*)).

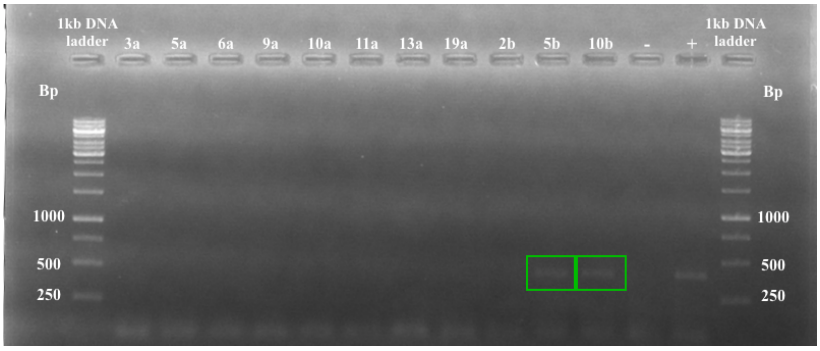

Figure 7. Agarose gel analysis of amplified *nifH* gene gene for 5b: *Pseudomonas asturiensis* and 10b: *Pseudomonas syringae.*

Relative abundance of isolated diazotrophs in total bacterial community

In order to determine the relative proportion of isolated diazotrophs in the total microbiome the 16S rRNA gene sequences of the diazotroph isolates were compared (megablast, e-value 0.003, min similarity 95%) with the sequences of a local database of the total microbiome from in-situ data of permafrost soils (Figure 8). Isolated diazotrophs in the soil sample covered approximately 2.5% of the total bacterial community in the dry site, 0.6% in the wet site, and 0.2% in the intact site. Among the diazotrophs, the genus *Paraburkholderia*, although originally isolated only from the dry site, appears to be the dominant genus on all the sites. Their proportion was approximately 0.86% in dry, 0.3% in wet and the least 0.05% in intact sites followed by *Peribacillus*, which was the second most dominant genus for the dry (0.08%) and wet (0.08%) sites but remained nearly undetected in undisturbed intact permafrost (Figure 8). *Mycobacterium* was the third highest proportion among the diazotrophs in all three sites: 0.41% for dry, 0.09% for wet and 0.042% for intact. *Peanibacillus* can also be seen to a larger extent of about 0.12% in the dry site, compared to the wet and intact sites, where their proportion was less than 0.001%. All conditions also display a certain proportion of *Pseudomonas, Pseudarthrobacter* and *Rhizobium* compared to the *Phyllobacterium* genus, whose proportion was completely undetected in dry and intact sites and very low in the wet site (Figure 8).

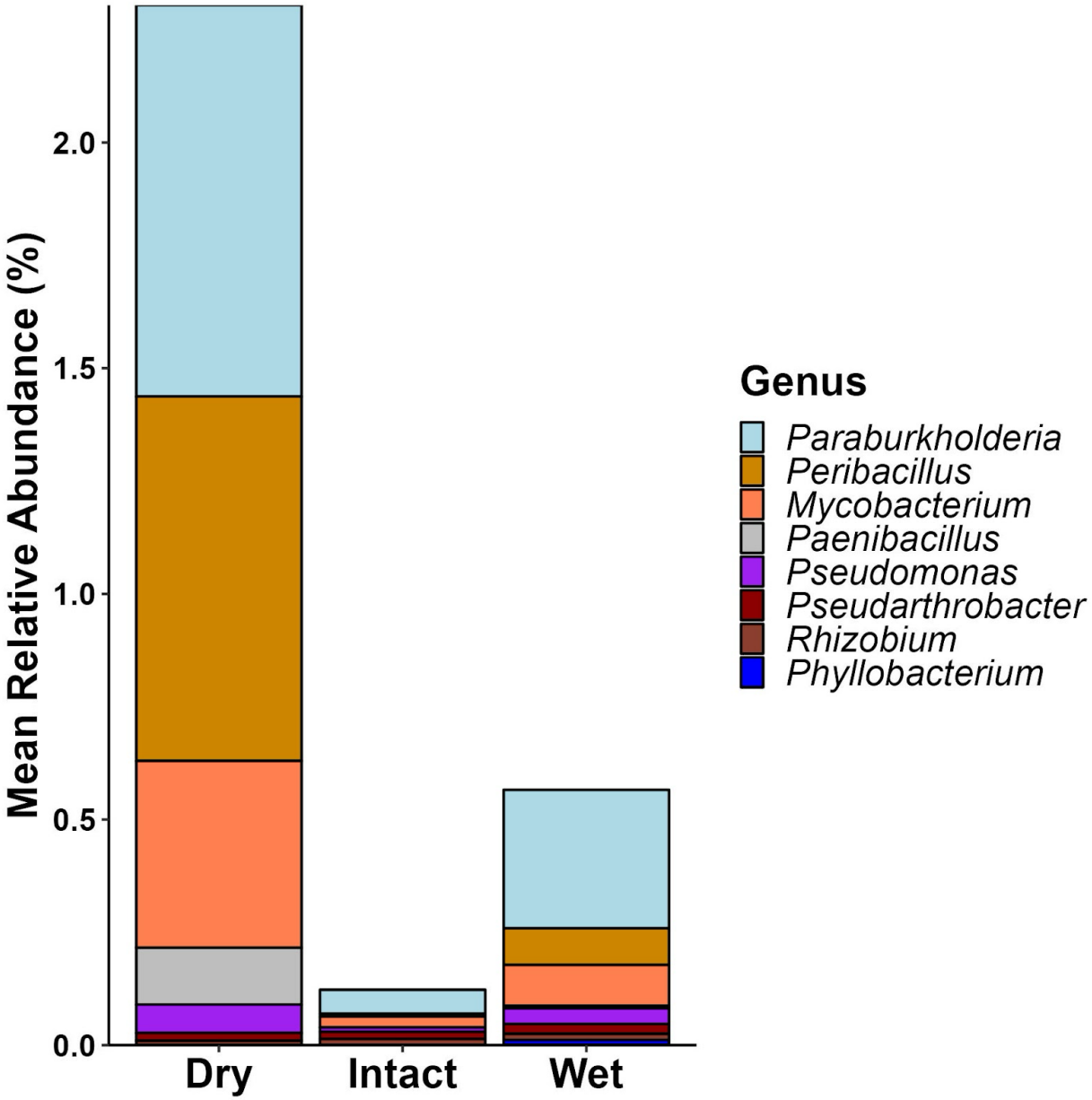

Figure 8. Relative abundance of diazotrophs at genus level in total bacterial community of three different sites of permafrost soil samples of 2021.

Microscopic analysis of diazotroph isolates

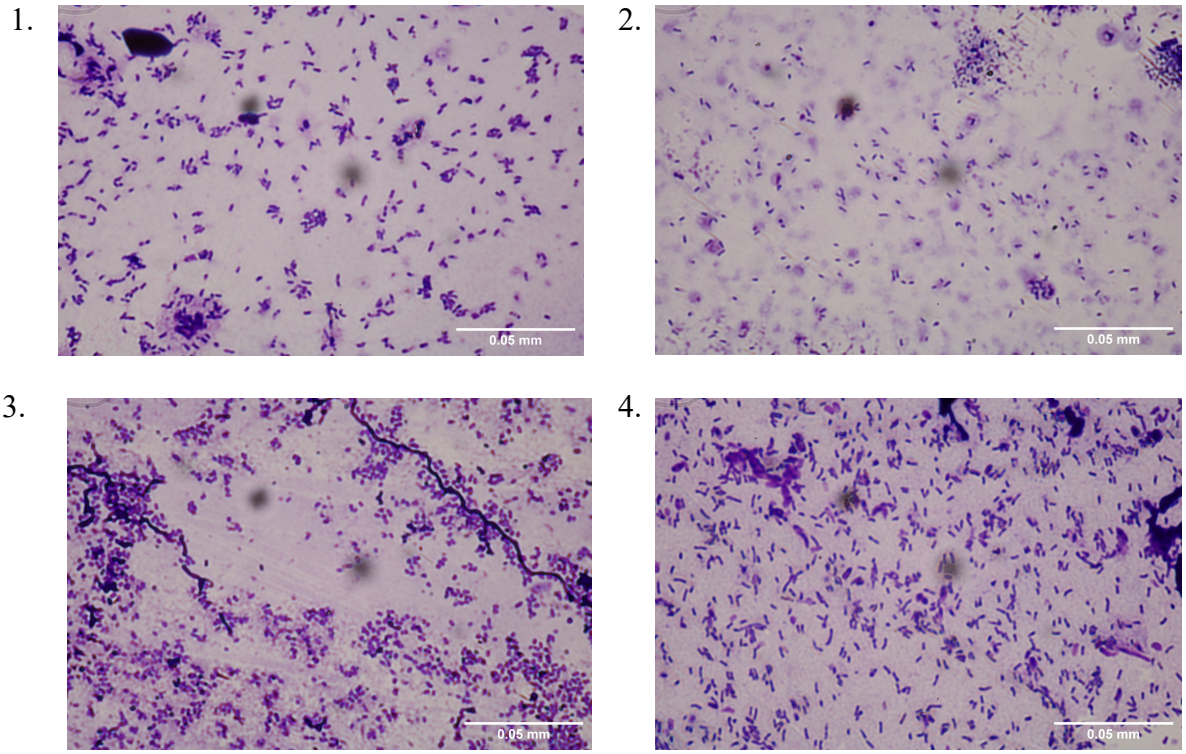

Figure 9. Microscopic photos of isolated bacteria 1. *Flavobacterium sp.*, 2. *Pseudomonas sp.,* 3. *Arthrobacter sp.,* 4. *Rhizobium sp.*

Discussion

Isolation and identification of diazotrophs

Overall, the abundance of diazotrophs in permafrost soils is low, for example, Siberian permafrost contains 10^7 - 10^8 of cells per g dry mass, while Antarctic permafrost soil contains 10^5 - 10^6 of cells per g dry mass, which corresponds to about 0.1% - 1% of the total cell community (Margesin & Miteva, 2011). Furthermore, there is a limitation to the culturability of cells in this type of soil, as many of the viable cells are non-culturable with unknown nutrient preferences (Oliver, 2005). This would explain why the CFU for the soil samples was low, as well as why there were only 33 distinct colonies that were further incubated.

Many of the genera cultivated in this study tend to contain diazotrophs, for example, for the most abundant genera in this study *Paenibacillus*, different species such as *Paenibacillus polymyxa* contain various diazotrophic strains (Lal and Tabacchioni, 2009). Furthermore, *Pseudomonas* and *Arthrobacter* contain diazotrophic strains that are also known to be psychrophilic (Kumar et al., 2019). The genus *Rhizobium* are also notable diazotrophs, often associated with plants and capable of living even in cold environments (Nash et al., 2018). Additionally, *nifH* gene-containing *Rhizobium* have previously been found in Alaskan permafrost, this study also investigated the impact of a thaw gradient on the differences between bacterial communities, however, this genus was not affected. These diazotrophs were identified using different *nifH* PCR primers: PolF/PolR. (Penton et al., 2016). The variability of genera containing diazotrophs could indicate that there were in fact more *nifH* carrying diazotrophs present amongst the samples, however they were not identified.

Although all isolates with sufficient DNA concentration were tested for the *nifH* gene, only three successfully showed the presence of this gene. One of the reasons for these findings could be due to primer binding issues. With any primer trying to bind a specific sequence in different species problems arise due to the variations of the *nifH* homologs among the species. In total, there are five groups into which nitrogenase-containing organisms tend to be separated (Gaby & Buckley, 2014). Due to this diversity, there are some groups in which the *nifH* gene will be missed by the primers (Angel et al., 2018). Another possibility for the lack of appearance of *nifH* bands on the gel is due to the low quality of the obtained DNA and its possible degradation. Many factors such as bead beater bead size, buffer type and temperature can affect the degree of DNA fragmentations during the extraction process (Bürgmann, Pesaro, Widmer & Zeyer, 2001), however, this should not be a problem with the DNeasy UltraClean Microbial kit, as it produces quite large fragments of 1 - 10kbp. On the other hand, compared to four other DNA extraction kits, the DNeasy has the lowest yield, which could reflect the low concentrations of extracted DNA in this experiment (Bogožalec, Lužnik, Tomič, & Milavec, 2023).

Since the first part of this thesis was the obtaining of 16S rRNA data, and the same collected DNA samples were used for *nifH* isolation later on, it is possible that due to the prolonged time of the experimentation, the DNA degraded and the primers could not properly bind the desired gene as

the sequence was no longer intact. This prolonged storage of DNA could also lead to potential contamination when it was initially used for the 16S rRNA isolation (May, 2018).

Additionally, not all bacterial samples were positive for the 16S rRNA PCR. One of the main reasons could be that some samples had very low DNA concentrations already upon extraction, therefore the PCR did not work and thus didn't produce any results on the gel. The low DNA concentration could be due to a loss of microbial matter during extraction, as some colonies were more difficult to remove from the agar gel than others, or the colonies were not that numerous. These could thus not be identified and associated with a genus or tested for *nifH* presence.

One of the isolates that were found to have the *nifH* gene, "*Kocuria"* (Order: Micrococcales), had the highest similarity with the species *Kocuria dechangensis,* however due to low similarity in the performed BLASTn it is possible it is an entirely different species as well as genus. This specifc species was first isolated and identified in 2015 from saline and alkaline soils in Dechang Township, China. Its colonies were beige-yellow in colour, and the bacteria were characterized as gram-positive, aerobic and coccus-shaped. The range of temperature at which they grew was from 4 °C - 50 °C (Wang et al., 2015). Instances of different species of this genus, such as *Kocuria rhizophila* can be found in permafrost soil (Afouda et al., 2020). The next isolate was *Pseudomonas asturiensis,* identified in 2013, described as gram-negative, aerobic and rod-shaped bacteria growing in beige colonies. They can grow in temperatures from 4 °C - 36 °C, even in 6% NaCl concentrations. They were first isolated from soybean plants in Asturias, Spain, where they were causing reddish spots on the leaves (González et al., 2013). Neither of the previously mentioned species was associated with being diazotrophic in literature. It could be that due to trimming the sequencing was not quite accurate and the isolates were mislabelled, or these are strains that were previously not identified as having the *nifH* gene. The last species isolated, *Pseudomonas syringae*, was mentioned in literature already in the early 20th century (Bos, 1903). It is known to be a very common pathogen that affects a wide range of plant species (Xin, Kvitko & He, 2018), however, there are instances of *P. syringae* being growth-promoting and diazotrophic, such as with the strain GR12-2, thus the strain isolated may also be a diazotrophic type of *P. syringae* (Patten et al., 2016).

Further investigating the microbial contents of the soil samples could still yield more diazotrophic strains. For example, due to the lack of anaerobic isolation conditions, there might be more diazotrophs present in the soil samples which were not capable of growth on the media that were exposed to regular atmospheric levels of O_2. To gain a deeper insight into the composition of the microbial community, it would be necessary to incubate plates in anaerobic chambers with N_2. A concentration of at least less than 2% of O_2 seems to be the standard for isolating a wider range of diazotrophs (Mirza & Rodrigues, 2012).

Relative proportion of diazotrophs in total bacterial community

For the majority of the genera, their proportions are similar between the wet and dry conditions, while in the intact the general presence of bacteria is quite low (Figure 8). However, there are some dissimilarities present, for example *Paenibacillus* is quite abundant in the dry soil condition but cannot be found in the others. Bacteria from this genus are found to be aerobic and often endophytic, so it is possible that the wet landscape with more anoxic habitats did not have suitable conditions (Johnson et al., 2021). Differences in conditions between the wet and dry soil could lead to unfavourable conditions for certain genera, such as with this example, the *Paenibacillus* could be able to thrive only in the oxic conditions of dry soil compared to the anoxic environment of wet soil. (Ping et al., 2015) Since there is a generally low abundance of nutrients (mainly N) in most permafrost soils, pH is a highly determining factor for the distribution of bacterial species (Ganzert, Bajerski & Wagner, 2014). This factor seems to be even more significant than dispersal limitation for the diversity of bacterial communities (Chu et al., 2010).

The most abundant from the samples investigated were *Paenibacillus* bacteria, they are known to be nitrogen fixers present in cold environments, which would reflect their high proportion (Varliero, Anesio & Barker, 2021). The other abundant genera in this study, *Mycobacterium* and *Paraburkholderia*, are also common permafrost dwelling genera (Singh et al., 2017).

Conclusion

The first aim of this study was to attempt to isolate various psychrophilic diazotrophs from active site permafrost soil under aerobic conditions. In total 33 bacterial colonies were extracted and inoculated, however only 25 were successfully sequenced for 16S rRNA. The second aim was to identify these bacteria using sequencing and investigate whether they contain the *nifH* gene. These sequenced bacteria were thus identified and split into 11 genera. Furthermore, three bacteria were also identified to carry the *nifH* gene, they belonged to 2 genera. These were "*Kocuria"* (Order: Micrococcales) (highest hit, but could be inaccurate, due to similarity less than 95%), *Pseudomonas asturiensis* and *Pseudomonas syringae*. For the third aim, the relative abundance of diazotrophs in the overall microbiome was obtained. Lastly, the data from this thesis was added to an overall database of the Cryovulcan project (GACR project n.20-21259J.) which studies the microbial biochemical activities, such as CO_2 production and degradation of organic matter in degraded permafrost sites. Mapping of diazotrophs in soil can further give insight into permafrost soil communities and the chemical processes within thawing permafrost soil.

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Appendix

Table A1. Amount of soil used for the isolation of diazotrophs

Type of soil	Grams used
Dry	2.03
Wet	2.01
Intact	2

Table A2. Colonies count on agar plates after incubation and CFU/g determination

Plate	First count	Second count	Total colonies	Mean	Grams of soil	CFU /g of soil
Wet R4 22°	33	41	74	76.66666667	2.01	37800
Wet R5 22°	28	42	70			
Wet R6 22°	36	50	86			
Imtact R4 22°	1	4	5	12	2	6000
Imtact R5 22°	3	18	21			
Imtact R6 22°	3	7	10			
Dry R4 22°	0	0	0	2	2.03	995
Dry R5 22°	1	2	3			
Dry R6 22°	1	2	3			

Remaining gels:

Figure A1. Agarose gel analysis for 16s rRNA gene implication

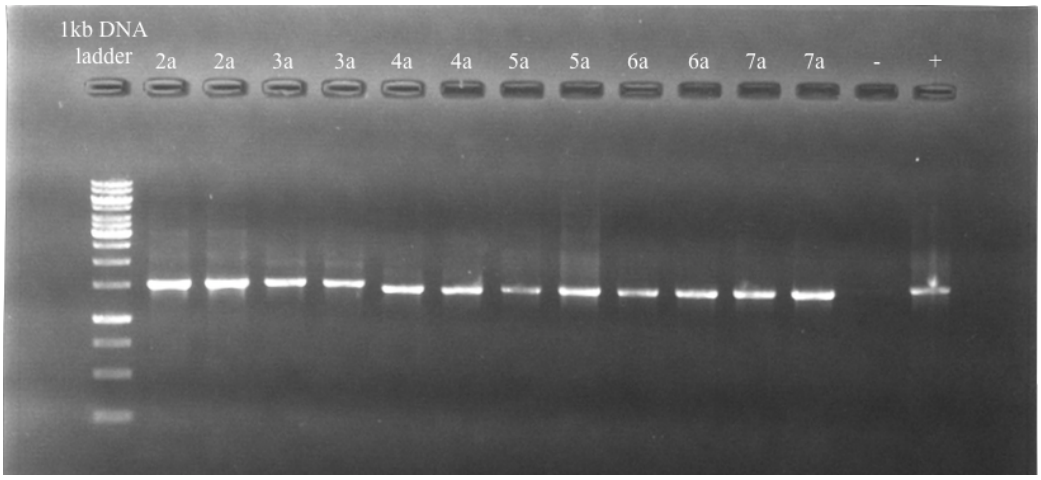


Figure A2. Agarose gel analysis for 16s rRNA gene implication.

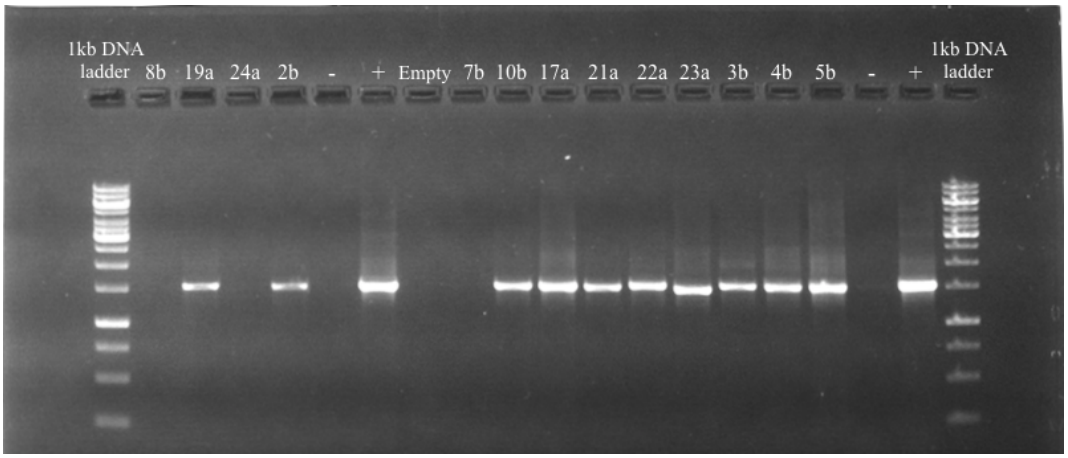


Figure A3. Agarose gel analysis for 16s rRNA gene implication

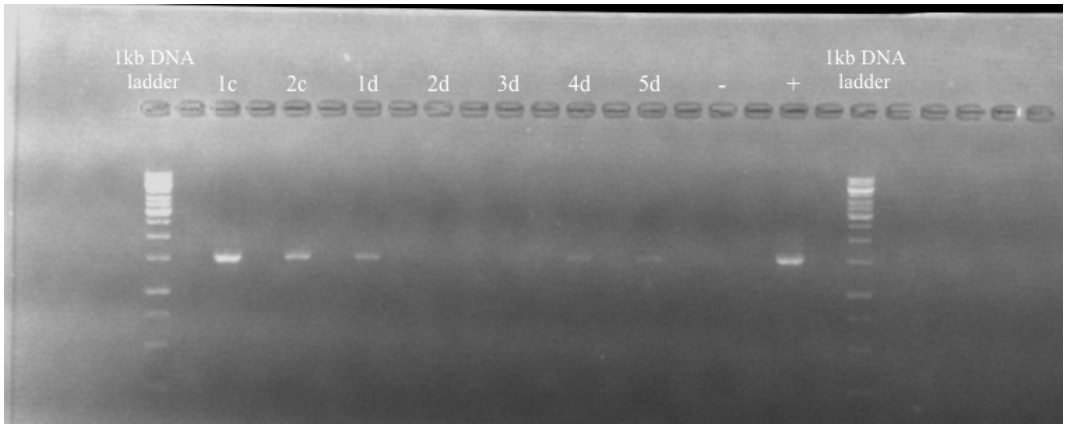


Figure A4. Agarose gel analysis for *nifH* gene implication

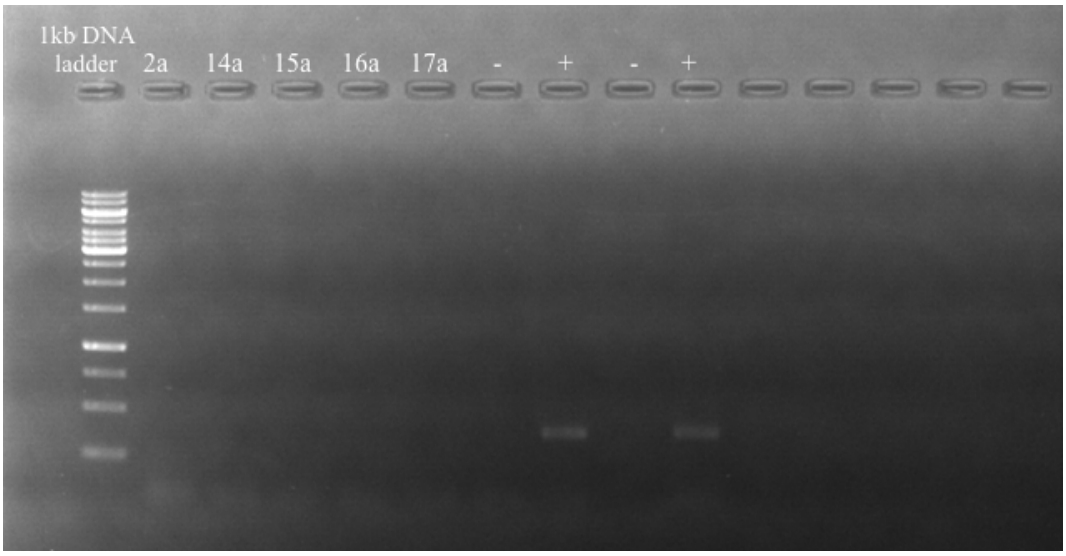


Figure A5. Agarose gel analysis for *nifH* gene implication

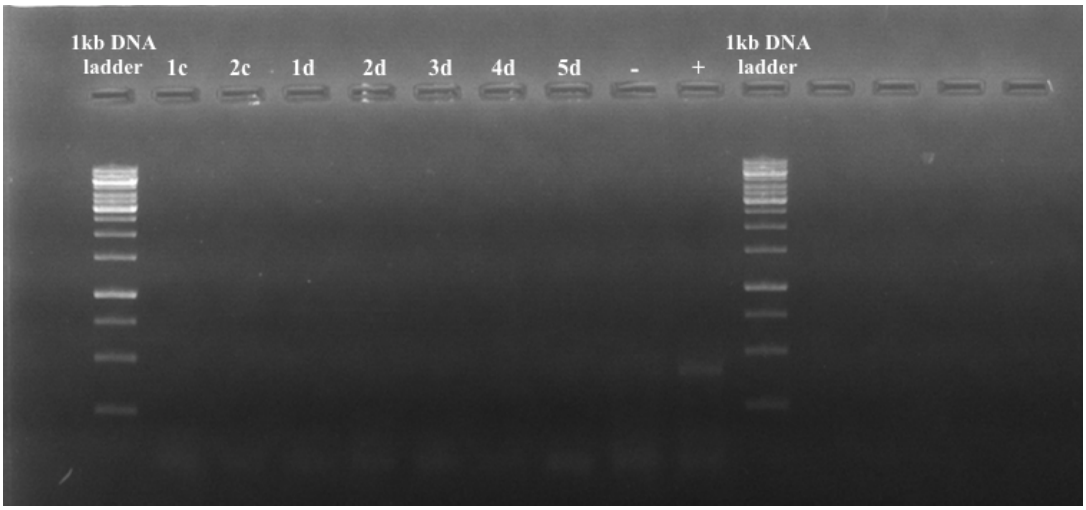
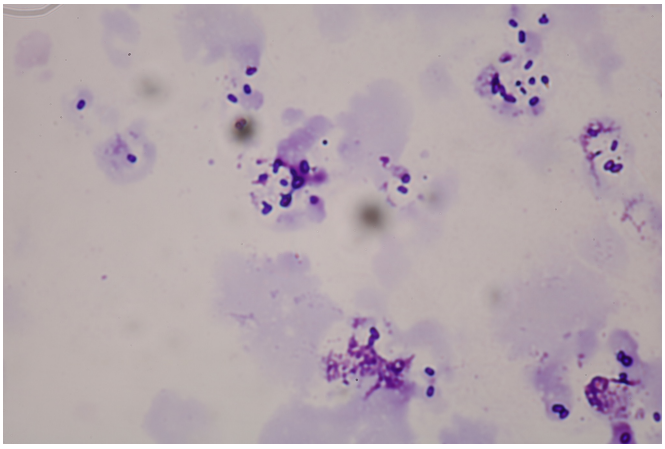
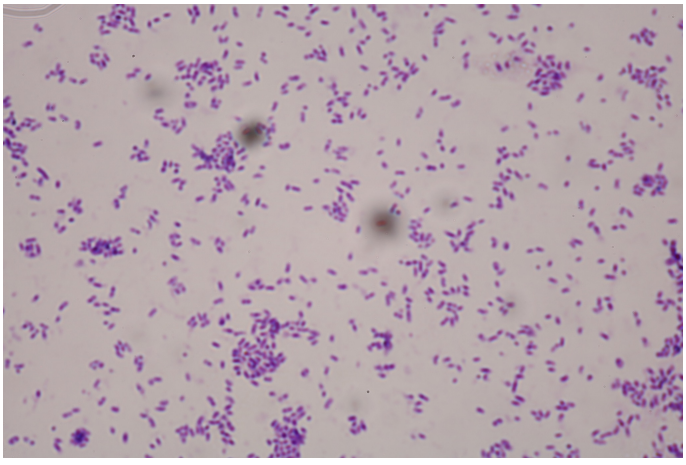


Figure A6. Microscopy photos of other genera:

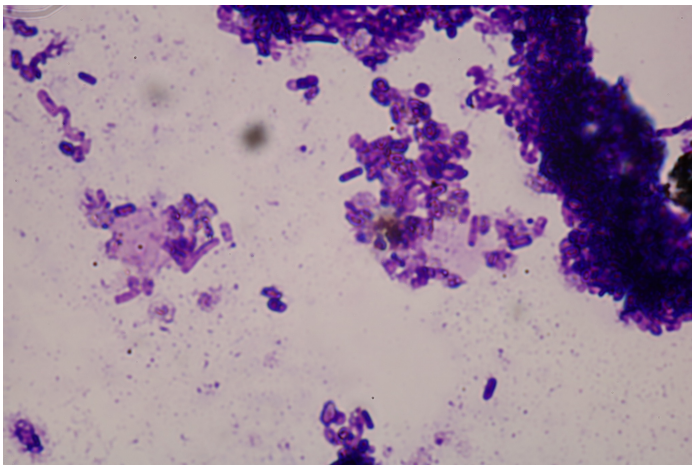
a. *Kocuria*



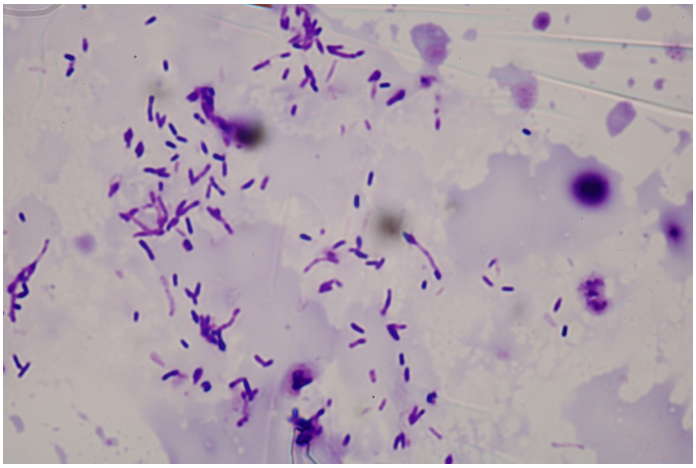
b. *Paraburkhorderia*



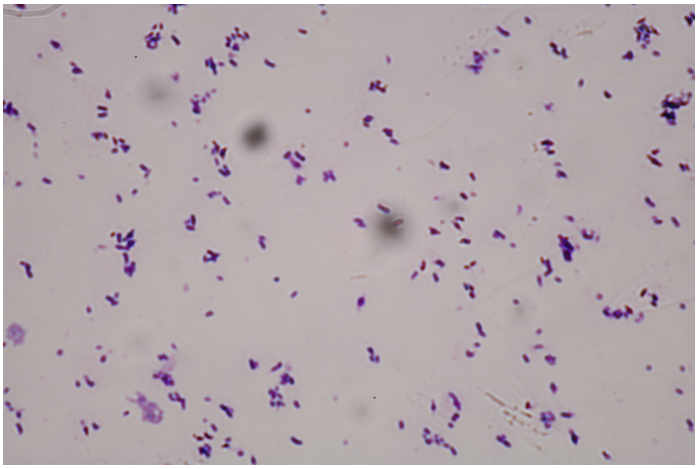
c. *Peribacillus*



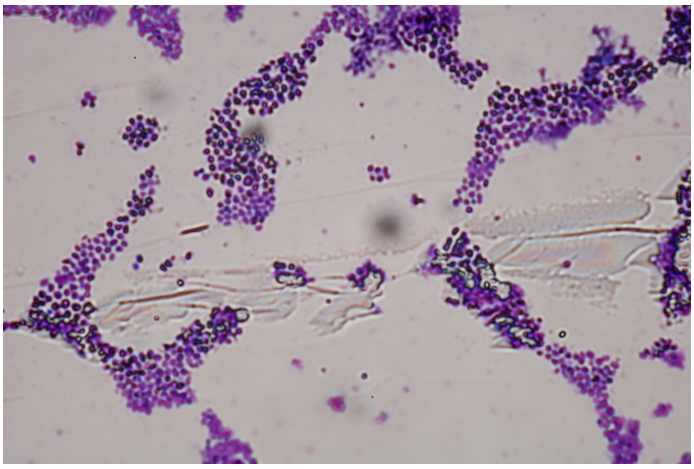
d. *Mycobacterium*



e. *Phyllobacterium*



f. *Pseudoarthrobacter*



g. *Paenibacillus*